

1 Long-term manure exposure increases soil bacterial community potential for plasmid
2 uptake

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14 Running title: Manure increases community plasmid uptake

15 **Abstract:**

16 Microbial communities derived from soils subject to different agronomic treatments were
17 challenged with three broad host range plasmids, RP4, pIPO2tet, and pRO101, via solid
18 surface filter matings to assess their permissiveness. Approximately 1 in 10,000 soil
19 bacterial cells could receive and maintain the plasmids. The community permissiveness
20 increased up to 100% in communities derived from manured soil. While the plasmid
21 transfer frequency was significantly influenced by both the type of plasmid and the
22 agronomic treatment, the diversity of the transconjugal pools was purely plasmid
23 dependent and was dominated by β - and γ -Proteobacteria.

24

25 **Text**

26 Rapid adaption of bacterial communities to changing environmental conditions is believed
27 to rely on lateral transfer of mobile genetic elements, such as plasmids, as one
28 indispensable mechanism (Sørensen et al., 2005; Thomas and Nielsen, 2005; Heuer and
29 Smalla, 2012). One of the crucial parameters that determines the extent of conjugal
30 plasmid transfer is community permissiveness, defined as the fraction of a microbial
31 community able to receive a given plasmid (Musovic et al., 2010). In agricultural soils,
32 seasonal application of manure and fertilizers provokes intense and immediate changes to
33 soil physical-chemical conditions that might modulate horizontal gene transfer (HGT).
34 Indeed, both an increased nutrient availability (van Elsas et al., 2003) and the introduction
35 of selective stressors like metals or antibiotics (Newby and Pepper, 2002; Heuer et al.,
36 2011) may lead to increased rates of plasmid transfer. Apart from this immediate
37 stimulation of HGT, it remains unknown whether agronomic soil treatment may have long-
38 term effects on permissiveness. To test this, we investigated permissiveness towards
39 three broad-host-range plasmids (Table 1) in four communities isolated from agricultural
40 soils subjected to different long-term seasonal treatments (Table 2).

41 Microbial communities from three plots (Untreated, Manured and Nitrate-Phosphate-
42 Potassium-fertilized (NPK)), at the long-term CRUCIAL experimental site (Taastrup,
43 Denmark) (Magid et al., 2006; Poulsen et al., 2013), were sampled (SI2) in order to assess
44 permissiveness. Treatments at the field site were in place for 8 years at the time of
45 sampling. The manure derived from a conventional dairy cow farm. The estimated
46 accumulative applications of C-N-P-K were estimated at 62800-3768-1184-2979
47 (manured), 0-840-120-400 (NPK) and 0-0-0-0 (untreated) kg/ha respectively (Magid et al.,
48 2006).

49 As reference, soil from an untreated plot of the well-known Rothamsted Park Grassland
50 (Rothamsted, United Kingdom) (Silvertown et al., 2006) site was included. From the
51 chosen soils, indigenous bacterial communities were isolated by Nycodenz®-extraction
52 (Musovic et al., 2010) and challenged with exogenous plasmids via solid-surface filter
53 matings (Musovic et al., 2010). *Pseudomonas putida* KT2442 (Bagdasarian et al., 1981)
54 served as the plasmid donor strain (Table 1). Conjugation events were detected by
55 epifluorescence microscopy, allowing their quantification (Musovic et al., 2010).

56 For all tested combinations, community permissiveness ranged from 8.24×10^{-5} to 4.56×10^{-4}
57 conjugations per recipient (Figure 1). Permissiveness was consistently higher (up to 3
58 times) for the soil-derived pIPO2tet plasmid than for the other two IncP plasmids, RP4 and
59 pRO101. Therefore, for both sampling sites, the introduced plasmid significantly influenced
60 the permissiveness ($p_{\text{CRUCIAL}} < 0.001$; $p_{\text{Rothamstead}} < 0.001$). Additionally, in CRUCIAL soils, a
61 significantly higher permissiveness was measured for the manure treated community
62 compared to the untreated control ($p_{\text{RP4}} = 0.041$; $p_{\text{pIPO2tet}} = 0.001$). Previous studies have
63 suggested that manure treatment may result in hot-spots of gene transfer due to increased
64 nutrient availability and cell density (van Elsas et al., 2003) with transfer frequencies
65 increasing by up to one order of magnitude (Götz and Smalla, 1997). However, this does
66 not explain why we observed stimulation of permissiveness by manuring because, in our
67 assay, heterogeneities in nutrient or cell density mating were excluded as matings were
68 carried out under standard nutritional conditions on soil extract medium (Musovic et al.,
69 2010). While raised levels of plasmid shuttled tetracycline resistance genes were shown to
70 occur in soils treated with piggery manure slurry (Agersø et al., 2006) or chicken waste
71 (You et al., 2012) in selective and enriching environments, this study is the first to indicate

72 that long-term manure treatment also changes the community permissiveness towards
73 newly introduced plasmids under neutral conditions.

74 Nutrient addition has previously been shown to enhance gene transfer frequency in soils
75 (Smets et al., 1995; Nielsen and van Elsas, 2001). To study the effect of fertilization, we
76 investigated the permissiveness of RP4 in the NPK-fertilized soil bacterial community.
77 Permissiveness in the NPK-treated soil was similar to the untreated control ($p=0.79$) and
78 significantly lower than in the manured one ($p=0.016$). Therefore, higher activity through
79 previous nutrient addition is not the reason for increased permissiveness in manured soil.
80 It thus seems that increased permissiveness was not due to immediate nutrient effects, or
81 increased cell density in hot-spots. However, increased permissiveness might have been
82 intrinsic to the community. Hence, we examined the diversity of the transconjugal pools, to
83 test whether they differ between soil treatments.

84 Random transconjugants were isolated from the matings using micromanipulation
85 (Musovic et al., 2010) to analyze, if apart from increased transfer frequencies, the
86 phylogenetic composition of the transconjugal pool changed due to long-term manure
87 application. Successfully isolated transconjugants were subjected to 16S rRNA
88 sequencing (Musovic et al., 2010). Sequences were analyzed using mothur v.1.30.0
89 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013). The sequences have
90 been submitted to GenBank and can be accessed under number KF590708 - KF591079.

91 Principal Coordinate Analysis (PCoA) revealed transconjugal pools separating primarily by
92 introduced plasmid (Axis 1, Figure 2). Independent of sampling site or treatment, pRO101,
93 RP4 and pIPO2tet were associated with significantly different transconjugal pools as
94 revealed by AMOVA (Excoffier et al., 1992) ($p=0.007$). Surprisingly, the number of OTUs

95 was lowest for pIPO2tet (SI Table 1), the plasmid exhibiting the highest permissiveness.
96 Although the effective phylogenetic host range in the tested soils appears smallest for
97 pIPO2tet, it might exhibit higher transfer frequencies, resulting in a higher total
98 permissiveness, possibly due to its nature as cryptic plasmid (van Elsas et al., 1998). High
99 transfer frequencies are especially important for the maintenance of those plasmids not
100 conferring any beneficial traits to their hosts.

101 The second dimension of PCoA separated all transconjugal pools from the CRUCIAL site
102 from those from Rothamsted ($p_{RP4} < 0.001$; $p_{pIPO2tet} < 0.001$). This separation likely derives
103 from differences in their original bacterial community composition. Meanwhile, an earlier
104 study – comparing deeply sequenced 16S rRNA community libraries – revealed no major
105 difference in the total bacterial community composition for different treatments at the
106 CRUCIAL site (Poulsen et al., 2013), hinting towards the observed grouping of those
107 transconjugal pools.

108 While plasmid and sampling site both affected transconjugal pools, no effect of agricultural
109 treatment was detected ($p > 0.9$) for any of the plasmids in the CRUCIAL soil. A closer look
110 at the six corresponding phylogenetic profiles (Figure 3) confirms that transconjugal pools
111 of the same plasmid within different soil communities are closely related. For instance,
112 *Enterobacteriaceae* sequences can only be found in both transconjugal pools associated
113 with pRO101, although their relative fractions differ. All six pools were dominated by β - and
114 γ -Proteobacteria. α -Proteobacteria, Flavobacteria and Sphingobacteria were detected in
115 lower abundance, revealing a wide variety of transconjugants (SI Table 1), in spite of the
116 low number of isolates. High-throughput analysis of transconjugal pools could in the future
117 lead to new insights in the extent of plasmid transfer in soils.

118 The similarity of transconjugal pools across soil treatments parallels that of the total
119 bacterial community composition of CRUCIAL soils (Poulsen et al., 2013). Therefore, the
120 increased community permissiveness in manured soil cannot be explained by difference in
121 community diversity. Increased seasonal nutrient availability can also be ruled out, since
122 high permissiveness was not observed for NPK-fertilized soil. Potentially, a higher
123 indigenous plasmid content in the community associated with manure applications (Marti
124 et al., 2013), can result in a higher permissiveness towards additional plasmids, by
125 increasing the mating potential of the plasmid bearing cells. Additionally, the introduction of
126 diverse plasmids through manure application may increase the community's
127 permissiveness. Indeed previous hosting of a plasmid has been shown to significantly
128 increase the permissiveness towards its renewed uptake in a *Dickeya* strain (Heuer et al.,
129 2010).

130 On the other hand, the periodic introduction of stressors, such as metal ions (Nicholson et
131 al., 1999) or antibiotics (Christian et al., 2003) present in manure, might lead to selection
132 mechanisms favoring more robust populations adapted to environmental changes through
133 increased permissiveness towards foreign DNA uptake (Heuer et al., 2008). These
134 selection mechanisms for more permissive strains do not have to be associated with
135 changes in the phylogenetic profile of the community, since permissiveness towards broad
136 host range plasmids of isolates that are genetically indistinguishable by 16S rRNA analysis
137 from identical field plot can differ by more than 2 orders of magnitude (Heuer et al., 2010).
138 Still, the exact mechanisms of the observed enhanced permissiveness are yet to be
139 elucidated. High-throughput analysis of the transconjugal pools could thus lead to insights
140 into community dynamics of plasmid transfer in soil.

141 Here, we confirmed that soil communities can serve as significant reservoirs for
142 exogenous conjugal plasmids. Approximately 1 in every 10,000 indigenous cells could
143 receive and maintain the studied plasmids. This frequency is increased up to 100% in soils
144 subject to manure treatment. Such an increase in the permissiveness of soil communities
145 and therefore in their potential for contributing to the spread of antibiotic resistance genes
146 is remarkable, making it crucial to elucidate the underlying mechanisms in further research
147 with higher sampling depth. Additional work investigating dissemination and mobilization
148 capacity of more diverse genetic elements among bacterial communities from differently
149 treated soils is therefore necessary.

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235 carrying plasmid in chicken-waste-impacted farm soil. *Appl. Environ. Microbiol.* **78**:
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237

238

239 Table 1: Plasmids^a carried by the red fluorescent donor strain, *Pseudomonas putida*
 240 KT2442 *DsRed::lacIq*

Plasmid	Inc-group	Type	Phenotype	Host range	References
RP4:: <i>Plac::gfp</i>	IncP-1α	Resistance	Tet ^R , Amp ^R , Km ^R	broad	(Barth and Grinter, 1977)
pIPO2tet:: <i>Plac::gfp</i>	(IncQ-mobilizer)	Cryptic	Tet ^R	broad	(Tauch et al., 2002)
pRO101:: <i>Plac::gfp</i>	IncP-1β	Catabolic	Tfd ⁺ , Hg ^R , Tet ^R ,	broad	(Harker et al., 1989)

241 ^aPlasmids are tagged using a TN5 cassette with a constantly expressed *gfp* gene that is
 242 *lacI* repressed in the donor strains.

243

244

245 Table 2: Origin of the recipient microbial communities

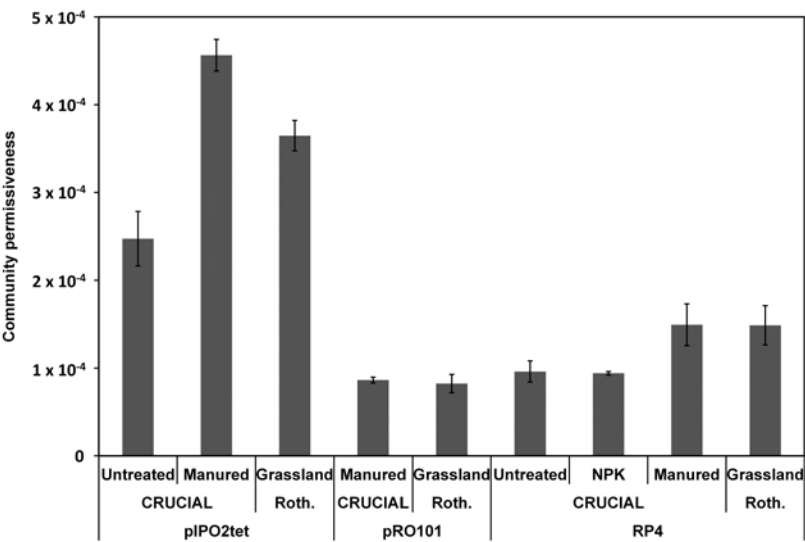
Soil origin	Soil type	Soil treatment	Coordinates	References
CRUCIAL Taastrup Denmark	agricultural	Untreated	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
		Manured	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
		NPK-fertilized	(55.681 N, 12.276 E)	(Poulsen et al., 2013; Magid et al., 2006)
Park Grass Rothamsted, UK	grassland	Untreated	(51.811 N, - 0.377 E)	(Silvertown et al., 2006)

246

247

248 Figure 1: Transfer frequency of the introduced plasmid to the soil indigenous bacterial
249 community after 48 hours of incubation on filters on soil extract medium. Values are
250 displayed as the mean of filter triplicates with standard deviation.

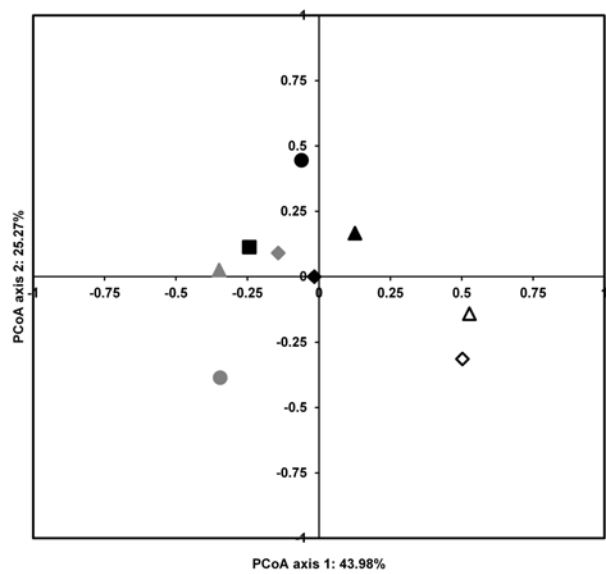
251



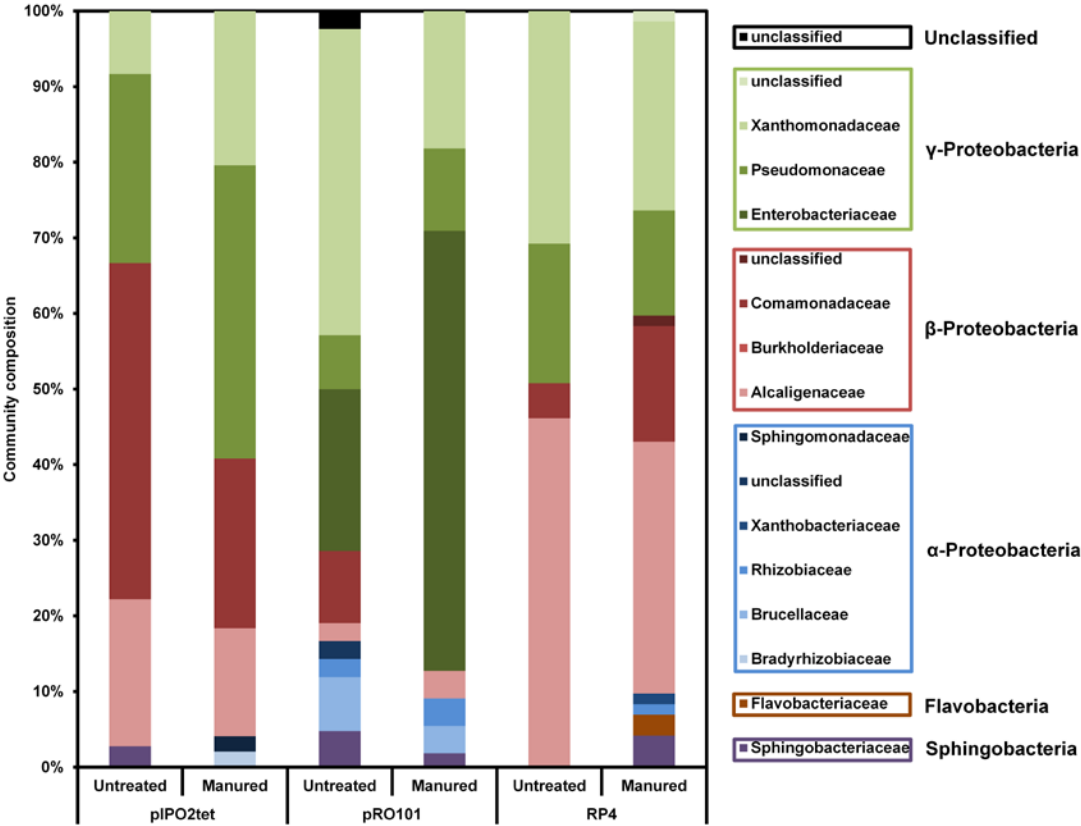
252

253

254 Figure 2: Principal Coordinate Analysis (PCoA) of the sequenced transconjugal pools.
255 Plasmids: pIPO2tet (red), pRO101 (green), RP4 (blue); Soil microbial communities:
256 CRUCIAL Manured (diamond), CRUCIAL Untreated (triangle), CRUCIAL NPK-fertilized
257 (square), Rothamstaed Park Grass (circle)



260 Figure 3: Phylogenetic analysis of the transconjugal pools originating from CRUCIAL
261 Manured and CRUCIAL Untreated soil microbial communities for all 3 tested plasmids.



264 SI Table 1: Diversity of transconjugants sorted by OTUs and occurrence per transconjugal
265 pool.

OTU	Kingdom	Phylum	Class	Order	Family	Genus	piPO2tet				pRO101		RP4			total sequences					
							Untreated	CRUCIAL	NPK	Manured	Rothamstead	Park Grass	Untreated	Manured	CRUCIAL		Manured	Rothamstead	Park Grass		
1	Bacteria	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	0	0	0	0	0	0	0	0	0	1	0	0	1			
2					unclassified	0	0	0	0	0	0	0	0	1	0	0	0	1			
3					Pedobacter	0	0	0	0	0	0	0	0	2	1	0	0	3			
4		Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium	1	0	0	0	0	0	0	0	1	0	0	2			
5							0	0	0	0	1	0	0	0	0	0	0	1			
6							0	0	0	0	0	1	0	0	0	0	0	0	1		
7		Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	unclassified	0	0	0	0	0	1	2	0	1	0	0	5			
8						Bradyrhizobiaceae	Bosea	0	0	1	0	0	0	0	0	0	0	0	1		
9						Brucecellaceae	Ochrobactrum	0	0	0	0	0	0	2	0	0	0	0	2		
10						Rhizobiaceae	Rhizobium	0	0	0	1	1	2	0	1	0	0	0	5		
11						unclassified	unclassified	0	0	0	0	1	0	0	0	0	0	0	1		
12						Xanthobacteraceae	unclassified	0	0	0	0	0	0	0	1	0	0	0	1		
13						Sphingomonadales	Sphingomonadaceae	Sphingomonas	0	0	1	0	0	0	0	0	0	0	0	1	
14						Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	7	9	7	2	1	2	27	21	4	0	80	
15									unclassified	0	0	0	0	0	0	1	1	0	0	0	2
16									unclassified	0	0	0	0	0	0	0	0	1	0	0	1
17									unknown	0	0	0	0	0	0	0	1	1	1	3	
18		Burkholderiaceae	unknown	0	0				0	0	0	0	0	0	0	5	5				
19		Proteobacteria	Enterobacteriales	Enterobacteriaceae	Comamonadaceae	16	0	11	0	4	0	3	11	0	0	45					
20					unclassified	0	0	0	0	0	1	0	0	0	0	1					
21					Pantoea	0	0	0	0	1	0	0	0	0	0	0	1				
22					unclassified	0	0	0	0	8	25	0	0	0	0	0	33				
23					unclassified	0	0	0	0	2	0	0	0	0	0	0	2				
24					unclassified	0	0	0	0	0	1	0	0	0	0	0	1				
25					unclassified	0	0	0	0	0	1	0	0	0	0	0	1				
26					unknown	0	0	0	0	0	3	0	0	0	0	0	3				
27					Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	8	4	18	1	2	5	11	10	2	0	61		
28								unclassified	0	0	0	0	1	0	0	0	0	0	0	1	
29		unclassified	0	0				0	0	0	1	0	0	0	0	0	1				
30		unclassified	0	0				0	0	0	0	1	0	0	0	0	1				
31		unclassified	0	1				0	0	0	0	0	0	0	0	0	1				
32		unclassified	0	0				1	0	0	0	0	0	0	0	0	1				
33		unclassified	0	0				0	0	1	0	0	0	0	0	0	1				
34		unclassified	1	0				0	0	0	0	0	0	0	0	0	1				
35		Xanthomonadales	Xanthomonadaceae	Stenotrophomonas				unclassified	0	3	4	0	1	0	18	7	10	0	43		
36								unclassified	3	4	6	0	6	8	1	11	3	0	0	42	
37					unclassified	0	0	0	0	4	0	0	0	0	0	0	4				
38					unclassified	0	0	0	0	0	2	0	0	0	0	0	2				
39					unclassified	0	0	0	0	1	0	0	0	0	0	0	1				
40					unclassified	0	0	0	0	0	0	1	0	0	0	0	1				
41					unclassified	0	0	0	0	4	0	0	0	0	0	0	4				
42					unclassified	0	0	0	0	1	0	0	0	0	0	0	1				
43					unclassified	0	0	0	0	0	0	0	0	0	0	0	1				
44					unclassified	0	0	0	0	0	1	0	0	1	0	0	1				
45		unclassified	0	0	0	0	0	1	0	0	0	0	0	1							
46		unclassified	0	0	0	0	0	1	0	0	0	0	0	1							
total OTUs							6	5	8	5	18	13	10	16	7	46					
total sequences							36	21	49	6	42	55	65	72	26	372					

266 SI Table 1: Diversity of transconjugants sorted by OTUs and occurrence per transconjugal pool. Sequences were analyzed using mothur v1.30.0 (Schloss et al., 2009) and the SILVA database (Quast et al., 2013).
267 These sequence data have been submitted to the GenBank database under submission ID 1656830.

268 SI 2: Sampling procedure and information

269 Soil samples of three different treatments were taken at the annually manured CRUCIAL
270 (Closing the Rural Urban Nutrient Cycle) agricultural field site (Taastrup, Denmark) (Magid
271 et al., 2006; Poulsen et al., 2013). Soil samples were collected in late fall 2010. Samples of
272 each treatment were taken from three different plots of this treatment. Each plot was
273 sampled for 1 kg of soil at 15 locations. The resulting soil volume was sieved and
274 homogenized to obtain a representative sample. Twenty grams of this homogenized soil
275 samples were used for Nycodenz®-extraction. The fourth sample was taken from the
276 monitored untreated Park Grass field in Rothamsted (Silvertown et al., 2006) (Rothamsted,
277 UK) in the exact same manner.

278